# Bioconversion of beetle-killed lodgepole pine to bioethanol

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# Abstract

Use of ethanol produced from biomass has the potential to offset use of fossil-derived fuels, reduce $CO_2$ emissions, and help reduce many effects of global warming, such as the current outbreak of the mountain pine beetle in British Columbia, Canada. This outbreak is increasing volumes of dead and dying lodgepole pine with time-limited commercial value. In this study, we focused on assessing the technical feasibility of producing ethanol from beetle-killed pine softwood. We pre-treated softwood with $SO_2$-catalysed steam or ethanol with subsequent enzymatic hydrolysis, and fermented the resulting sugars to ethanol. Both pre-treatments produced substrates from both beetle-killed and healthy lodgepole pine that could be hydrolyzed by cellulase enzymes more easily than could substrates produced previously from Douglas-fir wood. The receptivities of these substrates to subsequent enzymatic hydrolysis varied depending on the pre-treatment and conditions. Subsequently, methods were explored to characterize the substrates prior to hydrolysis, with the goal of gaining insight into the process parameters which may increase hydrolysis yields. Expertise and equipment at the University of British Columbia, such as the process development unit, were used to test the suitability of beetle-killed pine as a bioconversion feedstock.

Although further research is necessary to overcome various process bottlenecks in the overall bioconversion of beetle-killed pine, this study indicates that this resource could provide a significant source of biomass for bioconversion to ethanol.



**Keywords:** mountain pine beetle, lodgepole pine, bioconversion, cellulase, lignin, cellulose, hemicellulose, pre-treatment, hydrolysis and fermentation



# Résumé

L'utilisation de l'éthanol produit à partir de la biomasse a le potentiel de remplacer l'utilisation de combustibles fossiles, de réduire les émissions de $CO_2$ et de contribuer à réduire les nombreux effets du réchauffement planétaire, tels que l'actuelle infestation de dendroctone du pin ponderosa (DPP) en Colombie-Britannique (C.-B.), au Canada. Cette infestation est en train d'augmenter le nombre de pins tordus latifoliés morts ou ravagés, qui ont une valeur commerciale limitée dans le temps. Dans la présente étude, nous nous sommes concentrés sur l'évaluation de la faisabilité, sur le plan technique, de la production d'éthanol à partir de pins tordus latifoliés dévastés par le dendroctone. Nous avons utilisé un prétraitement à la vapeur catalysée par le $SO_2$ ou à l'éthanol, suivi d'une hydrolyse enzymatique, puis de la fermentation des sucres obtenus en éthanol. Les prétraitements à la vapeur et à l'éthanol ont produit des substrats à partir des pins tordus latifoliés dévastés par le dendroctone et des pins sains; ceux-ci ont pu être hydrolysés par des cellulases plus facilement que les substrats produits par le passé à partir de bois de Douglas taxifolié. La réceptivité des substrats produits par les processus de prétraitement à la vapeur et à l'éthanol à l'égard de l'hydrolyse enzymatique subséquente variait en fonction du type de prétraitement et des conditions employés. Par la suite, on a cherché des méthodes pour caractériser les substrats avant l'hydrolyse, afin d'en savoir plus sur les paramètres du processus qui peuvent provoquer une augmentation de la production de l'hydrolyse. Le savoir-faire et le matériel disponible à l'Université de la Colombie-Britannique, comme l'unité d'élaboration de procédés, ont été utilisés pour tester si les pins tordus détruits par le dendroctone pouvaient servir de matières premières de bioconversion.

Bien que des recherches plus approfondies soient nécessaires pour surmonter les difficultés liées aux différents procédés de bioconversion des pins tordus détruits par le dendroctone, l'étude indique que cette ressource pourrait fournir une source importante de biomasse pour la bioconversion en éthanol.



**Mots clés:** dendroctone du pin ponderosa, pin tordu latifolié, bioconversion, cellulase, lignine, cellulose, hémicellulose, prétraitement, hydrolyse et fermentation

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# Abbreviations

| | |
|---|---|
| AIL | Acid-insoluble lignin |
| ASL | Acid-soluble lignin |
| BK-CP | Beetle-killed pre-treated substrate centre point |
| EO | Ethanol organosolv pre-treatments |
| FPU | Filter paper units |
| HHF | Hybrid hydrolysis and fermentation |
| HMF | 5-hydroxymethyl furfural |
| IU | International units |
| PDU | Process development unit |
| SHF | Separate hydrolysis and fermentation |
| SSF | Simultaneous saccharification and fermentation |
| WIF | Water-insoluble fraction |
| WSF | Water-soluble fraction |

# 1 Introduction

Increased dependence on fossil fuels and consequent, potential impacts on climate change resulted in a global surge in bioethanol as a sustainable and renewable alternative. Bioethanol derived from lignocellulosic biomass in particular is promising as the utilisation of current starch-based feedstocks is constrained from a food security and environmental point of view. However, there is a need to ensure sufficient availability of lignocellulosic resources and appropriate conversion technologies in place to meet this ever-growing ethanol demand. The large volumes of mountain pine beetle-killed lodgepole pine in British Columbia may represent a large and potential resource for bioethanol. Although beetle-killed wood can, in the short term, be used for traditional lumber and pulp and paper applications, over time it loses its *structural integrity*, limiting its use for these applications.. However, using beetle-killed wood for bioethanol production would greatly help Canada meet its Kyoto greenhouse gas reduction targets while generating employment in rural areas. Producing bioethanol-based fuels and blends at competitive prices is a main strategy of Brazil, Sweden, and the US, among other countries, to decrease their dependence on imported oil and to decrease greenhouse gas emissions and reduce the environmental impacts of the transport sector (Mabee et al. 2005). Developing alternative fuels is of even greater importance, given the news of Canada's recent inability to meet its goals for $CO_2$ emission reductions (CBC 2006).

Lodgepole pine is one of the most abundant tree species in British Columbia, dominating the interior forest regions. The current devastation to lodgepole pine forests created by the mountain pine beetle epidemic has resulted in an increase in harvesting activity to make use of infested lodgepole pine trees before they lose their commercial value as lumber or pulp. The estimated *shelf life* of beetle-killed wood for structural applications varies depending on several environmental factors. Degradation can range from 1-15 years before fibre loss, decay, and checking (Trent et al. 2006) and potentially reduce its commercial value. At the same time, if left standing in the forest, the dry trees pose a potential fire hazard for surrounding communities. If technically feasible, converting beetle-killed pine into bioethanol may be an ideal value-added application for this material.

The bioconversion of lignocellulosic feedstocks to ethanol consists of three major steps:

1) pre-treatment to overcome the recalcitrance of the cellulosic substrate to
2) subsequent enzymatic hydrolysis by carbohydrate degrading enzymes to create monomeric sugars which are
3) subsequently fermented to ethanol.

One of the major technoeconomic issues that has arisen during lignocellulosic bioconversion research is the search for an economically effective pre-treatment for opening up the complex structure of biomass for subsequent bioconversion. The pre-treatment step is recognized as carrying particular importance, as it affects both the upstream selection of biomass; the ability to recover the cellulose, hemicellulose and lignin components in a usable form; and the properties of the resulting substrate which govern downstream hydrolysis and fermentation. Thus, an effective pre-treatment should be economical, robust, recover most of the lignocellulosic components, and provide a cellulosic substrate that can be efficiently hydrolyzed by a low enzyme input.

Typically, softwood-derived biomass such as lodgepole pine is tougher to process than hardwood and non-woods due to its higher lignin content, coarse fibre structure and the greater degree of cross-linking within the lignin macromolecule (generally thought to be due to a higher proportion of guaiacyl lignin subunits). During hydrolysis, lignin can act as both a steric barrier, restricting access to cellulases, and as a non-productive adsorbent to cellulases, thus inactivating the enzymes during substrate hydrolysis (Mansfield et al. 1999; Berlin et al. 2006). Therefore, softwood pre-treatment requires further development to achieve effective hydrolysis and fermentation. The University of British Columbia Forest Products Biotechnology Group has studied both steam pre-treatment and ethanol organosolv (EO) pre-treatment as potential technologies for processing woody lignocellulosic substrates such as softwoods and

hardwoods. Ethanol organosolv pre-treatment was originally developed as a pulping process for the pulp and paper industry (Kleinert 1974). A mixture of water with an organic solvent such as ethanol and an acid catalyst (HCl or $H_2SO_4$) partially removes lignin and resulting in a cellulose-rich substrate which can be subsequently hydrolyzed by cellulases. However, one potential challenge for EO is the recovery of the hemicellulose fraction which remains in the lignin-rich ethanol:water product stream. During the EO process, the solvents can potentially be evaporated, condensed and recycled, while the lignin can be isolated in a relatively pure form. Steam pre-treatment of biomass frequently utilizes sulphur dioxide in a process referred to as $SO_2$-catalyzed steam pre-treatment. The process consists of subjecting the feedstock to high-pressure steam at temperatures of 200° C–240°C for retention times ranging from 20 seconds -5 minutes, concluding with a final pressure release (explosion) that facilitates the disintegration of the feedstock. Impregnating biomass with $SO_2$ prior to steam pre-treatment affords shorter reaction times at lower temperatures and results in a solid fraction having enhanced enzymatic hydrolysability and liquid fraction containing the hemicellulose-derived sugars mostly in monomeric form. Recovery of hemicellulose is important for softwoods in particular as its hemicellulose is rich in hexose sugars which could be relatively easily fermented to ethanol with conventional microorganisms such as *Saccharomyces cerevisiae* (Hahn-Hägerdal et al. 1994). In this project, we focused on both the EO pre-treatment and steam pre-treatment processes due to their potential to process softwood biomass.

The project was developed to explore the potential for bioconversion processes to effectively utilise the abundant availability of beetle-killed pine in British Columbia to produce fuel and bioproducts. Bioconversion typically involves pre-treating the biomass to improve accessibility of the hydrolytic enzymes to the carbohydrate component for hydrolysis, then fermenting the resulting monomeric sugars to ethanol. These processes may be carried out separately or in combination. As mentioned earlier, although all three steps are important, pre-treatment has a considerable influence on the other two *downstream* processes and also on the upstream selection of biomass. For example, severe pre-treatment conditions result in considerable degradation of sugars in the liquid fraction, thus compromising fermentation performance, while mild conditions result in a solid fraction less amenable to enzymatic hydrolysis. Although various methods have been explored for their potential to process biomass (Mosier et al. 2005), steam pre-treatment and EO pre-treatment methods have been shown to be effective in processing softwood biomass (Brownell et al. 1986; Pan et al. 2007).

Pre-treatment using $SO_2$-catalyzed steam pre-treatment has been applied to many softwood species with varying levels of success. In particular, Douglas-fir (*Pseudotsuga menziesii*) has been a focus of the research of our group for the past 15 years (Brownell et al. 1986; Wu et al. 1999; Boussaid et al. 2000). Although optimised pre-treatment conditions for Douglas-fir could result in significant hemicellulose recovery in the liquid stream, delignification of the resulting solid fraction in a post-treatment step was still necessary to obtain acceptable hydrolysis yields (Clark and Mackie 1987; Wong et al. 1988)

The organosolv pre-treatment process employing ethanol as the solvent and has been investigated largely from the perspective of pulp production, with only a few groups investigating its potential for bioconversion. Our group has previously shown that the EO process results in a liquid fraction with relatively reactive lignin fraction with limited carbohydrate dissolution while obtaining a solid fraction having high amenability to enzymatic hydrolysis at relatively low enzyme input (Pan et al. 2006). Recently, we applied organosolv pre-treatment over a range of conditions to poplar variants, carrying out subsequent mass balance and enzymatic hydrolysis. Pre-treatment conditions were found to influence both the sugar recovery and the ease of enzymatic hydrolysis of the resulting substrate. Later studies evaluated the resulting lignin fractions for their polymer and antioxidant properties and the cellulosic fractions for their physiochemical properties (Pan et al. 2006). We have also assessed the ability of EO pre-treatment to process mixed softwood biomass samples (spruce, pine, and fir mix). The process produced a substrate with superior enzymatic digestibility over those pre-treated by other methods.

The current project assessed the potential of two promising pre-treatments, organosolv pre-treatment and steam pre-treatment, for their ability to process beetle-killed and healthy trees, and examined the issues at

each stage of the downstream processing of the substrates (subsequent hydrolysis and fermentation steps) to ethanol. The project also developed methods to assess the effectiveness of pre-treatment in producing substrates which were more amenable to subsequent hydrolysis by cellulases. (Yang et al. 2002) showed that it is difficult to obtain a substrate that can be hydrolyzed by cellulases at relatively low enzyme loadings without applying a post-treatment step to remove the lignin component of the steam-pre-treated substrates. The substrate factors resulting from pre-treatment that enable enzymatic hydrolysis still need to be fully resolved (Chandra et al. 2007). Substrate characteristics imparted by pre-treatment apparently influence the efficiency of enzymatic hydrolysis considerably, likely because pre-treatment influences both the substrate's chemical constituents (cellulose, hemicellulose, and lignin) and its morphological/ physical features such as crystallinity, degree of polymerization, and accessible surface area which can all play a key role in the ability of a cellulase enzyme to hydrolyze the respective substrate.

The surface area of a substrate accessible to cellulases may be one of the most influential factors affecting hydrolysis, as the enzyme requires contact with the substrate to hydrolyze it. Without pre-treatment, hydrolytic enzymes (cellulases) have limited access to the cellulose within the matrix of lignin and hemicellulose (Chandra et al. 2007). A major issue facing research into the interaction of cellulases with pre-treated softwood substrates to improve the overall process is the ability to measure the actual area accessible to cellulases. Most measurement techniques require drying the substrate, which causes its innate surface area to decrease. Measurements which can assess substrates in the *wet-state* have mainly evolved from techniques to evaluate pulps for papermaking such as water-retention value and Simons' staining (Chandra et al. 2007). Therefore, one of the project's goals was to evaluate and improve existing methods that predict the relative ease of hydrolysis of pre-treated lodgepole pine substrates. A relatively quick method for evaluating the susceptibility of pre-treated substrates to enzymatic hydrolysis without having to perform subsequent hydrolysis would optimize pre-treatment processes and save enzyme use and time, since it can take 12–72 h to determine the hydrolysis reaction's rate and extent.

Another element of the bioconversion process in need of improvement, especially considering the recalcitrance of softwood-derived biomass feedstocks such as lodgepole pine, is the ability to perform effective hydrolysis reactions at increased solids loadings. A higher solids loading could allow for increased concentration of the sugar stream supplied to fermentation, thus decreasing the energy required for the distillation of ethanol. Unfortunately, conducting hydrolysis under medium to high solids conditions (10%–30%) presents significant challenges due to the energy and equipment required to mix a highly viscous substrate suspension, and the consequent possibility of shear inactivation of cellulases (Gunjikar et al. 2001). At higher solids loadings, the build-up of hydrolysis products can result in slowing or stalling the enzymatic hydrolysis reaction due to end-product inhibition (Todorovic et al. 1987). Therefore, we evaluated the hydrolysis results at elevated solids loadings at the flask scale, using pre-treated lodgepole pine substrates from the initial phases of the project, to potentially improve hydrolysis.

The project initially involved evaluating steam and EO pre-treatments for processing beetle-killed pine and healthy lodgepole pine substrates in a pre-treatment, hydrolysis, and fermentation scheme. The optimal pre-treatment conditions identified above were applied to generate substrates for further testing. Hydrolysis/fermentation process configurations, such as separate enzymatic hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF), were tested and compared.

Using the optimal feedstocks of both healthy lodgepole pine and beetle-killed pine, water-washed insoluble fractions from both pre-treatments were enzymatically hydrolysed to determine their enzymatic digestibility with the use of cellulolytic enzymes at low enzyme loadings. The water-soluble fractions (WSFs) obtained from the pre-treatment/hydrolysis steps were collected to assess their efficiency in the fermentation to ethanol by yeast (*Saccharomyces cerevisiae*). Subsequent studies were aimed at the development/application of substrate assessment techniques for pre-treated lodgepole pine substrates to possibly aid process optimization to lower applied enzyme loadings for enzymatic hydrolysis, and to improve the hydrolysis of pre-treated beetle-killed pine/lodgepole pine substrates at elevated solids loadings. Much of the reported work focused on the interface between the pre-treatment and hydrolysis

processes of bioconversion, since these processes were found to carry particular significance in maximizing the overall sugar production from pre-treated beetle-killed pine/lodgepole pine feedstocks for subsequent fermentation to ethanol. Hydrolysis of pre-treated beetle-killed pine substrates were compared to pre-treated Douglas-fir substrates from a single sample (a 168-year-old Douglas-fir), a softwood substrate which has been historically recalcitrant to pre-treatment and hydrolysis.

This study determines the feasibility of utilizing beetle-killed pine for bioethanol production by employing steam and ethanol pre-treatment, as well as both separate and simultaneous hydrolysis and fermentation. The objectives of the project can be further broken down into the following sub-tasks.

1) Optimize steam and ethanol-organosolv pre-treatment conditions that provide the highest potential ethanol yield from beetle-killed pine. As beetle-killed pine behaves differently from healthy lodgepole pine, due to changes in moisture content and chemistry, we optimized both pre-treatment processes for beetle-killed pine.

**Performance measure:** Percent conversion of beetle-killed pine to monomeric sugars after pre-treatment and subsequent enzymatic hydrolysis

2) Evaluate robustness of optimised pre-treatment conditions on healthy lodgepole pine. Since future feedstock supplies in British Columbia are likely to be a mixture of healthy lodgepole pine and beetle-killed pine, pre-treatment conditions must be sufficiently robust to provide comparable ethanol yields from both feedstocks.

**Performance measure:** Percent conversion of healthy lodgepole pine to monomeric sugars after pre-treatment and subsequent enzymatic hydrolysis

3) Compare the effectiveness of SHF and SSF processes to convert pre-treated beetle-killed pine and healthy lodgepole pine substrates to ethanol.

**Performance measures:** 1) Overall ethanol yield within a reasonable period of time (72 hours)

4) Develop and assess laboratory tests which will enable further elucidation of the substrate factors that affect the enzymatic hydrolysis of pre-treated substrates.

**Performance measures:** Correlation of the degree of substrate accessibility (determined by the developed methods) to cellulolytic hydrolysis yields

# 2 Materials and Methods

## 2.1 Materials

### 2.1.1 Raw materials

The beetle-killed pine used for this study was carefully selected to represent the wide variation in characteristics that occurs over an area as large as British Columbia. Similarly, healthy wood was selected which had as many characteristics as possible in common with the beetle-killed wood, primarily age and diameter. Beetle-killed pine was provided by our colleague at the University of British Columbia, Dr. Collette Breuil, in the form of 14 bolts of wood from upper and lower sections of seven grey-phase trees attacked and killed three years prior to harvest in central British Columbia. The average age of the trees was 99 years. Healthy samples came from bolts of wood from the lower sections of four non-beetle-attacked lodgepole pines in south–central British Columbia, provided by Ken Ewanick at Tolko Industries Ltd. in Vernon, British Columbia. The average age of these trees was 101 years. Both sets of samples were debarked, split, chipped, and screened to approximately 2 × 2 × 0.5 cm. Chips from the seven beetle-killed trees were pooled, as were chips from the four healthy trees. The final moisture content of the chips was 12% weight for weight (w/w) for the beetle-killed pine and 11% w/w for the healthy lodgepole pine. Composition of the raw feedstocks (replicate) is shown in Table 1. The Douglas-fir biomass employed for all experiments was obtained from a 150-year-old Douglas-fir tree obtained from the University of British Columbia Research Forest and was approximately 155 years old.

**Table 1**. Chemical composition of wood chips used in the project.

| Wood Substrate | Arabinan | Xylan | Glucan | Galactan | Mannan | AIL[a] | ASL[a] |
|---|---|---|---|---|---|---|---|
| healthy lodgepole pine | 1.9 | 6.2 | 44.9 | 3.0 | 11.5 | 26.2 | 0.4 |
| beetle-killed pine | 1.7 | 5.9 | 41.9 | 2.7 | 11.2 | 28.5 | 0.6 |
| Douglas-fir | 1.0 | 3.4 | 44.6 | 2.5 | 12.5 | 31 | 0.5 |

### 2.1.2 Steam pre-treatment

Experiments were conducted once due to time and equipment constraints, with all samples in duplicate unless otherwise indicated. As a result, all error bars shown in figures are based on the deviation of the duplicates from the mean. The solid particles produced after pre-treatment were quite varied in size due to the large variation in size of the wood chips in the starting material. The insoluble fraction after pre-treatment was not screened to remove oversized chunks, which caused some variability between duplicates in each experiment and produced a larger range of results. Such error was usually larger at the beginning of the experiment, while by the end any larger particles which were slower to hydrolyse would have been hydrolysed to their full extent and, subsequently, the differences between duplicates were reduced. Ethanol yields were calculated as the percent of theoretical ethanol yield given that the yeast *Saccharomyces cerevisiae* produces 0.51 g of ethanol per g of hexose under ideal conditions (Hahn-Hägerdal et al. 1994). Experimental yields were calculated based on the fermentable sugars available in the pre-treated materials at the start of the experiment. Overall yields were calculated based on the initial concentration of hexoses in the starting biomass.

Prior to steam pre-treatment, wood chips were impregnated with sulphur dioxide by adding gaseous $SO_2$ (Praxair Canada) to plastic bags containing 300 g dry weight of wood chips. The bags were weighed and left at room temperature overnight. In the morning, any excess gas was released and the bags were reweighed to measure how much acid had been absorbed. An average of 78% of the $SO_2$ was absorbed by the beetle-killed wood and 95% by the healthy. The impregnated chips were added to the reactor of a 2-L StakeTech II steam gun (Stake Technology, Norval, ON) in 50 g d.w. portions and treated at the specified temperature. After 300 g d.w. had been discharged to the collecting vessel, the slurry was removed and stored at 4° C. After the bulk of the material had been removed, the vessel was rinsed with tap water and the liquid collected separately and analyzed for sugars to improve the closure of the mass balance. The WSF was separated from the water-insoluble fraction (WIF) by vacuum filtration. The WIF was then washed with a volume of water twenty times the dry weight. Monomeric and oligomeric sugar concentration were determined for the WIF wash liquid, steam gun wash liquid, WSF, and WIF to calculate the sugar recovery.

### 2.1.3 Organosolv pre-treatment

In brief, MPB lodgepole pine chips were pre-treated in aqueous ethanol with sulfuric acid as a catalyst using a custom-built, four-vessel (2 L each) rotating digester made by Aurora Products Ltd. (Savona, BC). A 200 g (oven-dried weight) batch of chips was pre-treated in each vessel. Vessels were opened after being cooled to room temperature in a water bath. Spent liquor (i.e., aqueous ethanol in the vessel) was sampled immediately to determine furfural, HMF, and formic and levulinic acids. The substrate (i.e., defiberized solid fraction) and spent liquor were then separated using nylon cloth. The substrate was washed three times (300 mL each) with warm (60° C) aqueous ethanol having the same concentration as the pre-treatment liquor. The washes were combined with the spent liquor. The substrate was then washed three times with water at 60° C, and the washes were discarded. The washed substrate was homogenized in a standard British disintegrator for 5 min and passed through a laboratory flat screen with 0.008-in. (0.203-mm) slits (Voith Inc., Appleton, WI) to remove rejects (i.e., non-defiberized woodchips and knots). The yields of rejects and screened substrate were determined. The screened substrate was stored at 4° C for analysis and hydrolysis. The spent liquor and the ethanol washes were combined and mixed with three volumes of water to precipitate the dissolved lignin. The lignin precipitate, henceforth denoted as

EO lignin, was collected on Whatman No. 1 filter paper, washed thoroughly with water, and air dried. he filtrate and the water washes were combined to give a WSF containing monomeric and oligomeric saccharides, depolymerized lignin, and compounds derived from saccharides.

### 2.1.4 Enzymatic hydrolysis

Hydrolysis experiments were conducted in Erlenmeyer flasks with washed solids enzymatically hydrolysed at 2% (weight/volume) consistency in acetate buffer (50 mM, pH 4.8) at 50° C and 150 rpm. Enzymes were added in the form of cellulase at the specified enzyme loading and β-glucosidase at the specified loading. At specified times over 48 h, 400 µL samples were taken, boiled for 5 min, and stored at –20° C.

### 2.1.5 Analysis of carbohydrates and lignin

Monosaccharides were measured on a Dionex HPLC (high-performance liquid chromatography) equipped with an anion exchange column (Dionex, CarboPac PA1). Deionised water at 1 mL/min was used as an eluent, and postcolumn addition of 0.2 M NaOH maintained baseline stability and detector sensitivity. After each analysis, the column was reconditioned with 1 M NaOH. Injections of 20 µL of each sample were administered after filtration through a 0.45 µm syringe filter (Chromatographic Stream pre-treatment specialities, Brockville, Canada). Standards were prepared containing sufficient arabinose, galactose, glucose, xylose and mannose (all Sigma) to encompass the same range of concentrations as the samples. All samples and standards received 0.2 g/L fucose (Sigma) as an internal standard. Post-hydrolysis analysis of all liquid samples allowed quantification of the amount of oligomeric sugars present. An addition of 0.7 mL of 70% $H_2SO_4$ was made to 15 mL of the liquid sample and the volume made up to 20 mL with water. Samples were autoclaved at 121° C for 1 h and analyzed by HPLC as described above. Solid samples were analyzed for insoluble (Klason) lignin and sugars using the modified Tappi T-222 om-88 method (Tappi-standard 1998). Briefly, the raw wood or pre-treated material was air dried and ground to pass through a 40-mesh screen. The ground sample (0.2 g) was mixed with 3 mL of 72% sulphuric acid and incubated at 20°C for 2 h and stirred every 10 min. Following the acid hydrolysis, the reaction mixture was diluted with 112 mL of deionised water for a total volume of 115 mL and 4% acid concentration. The solution was transferred to a serum bottle and autoclaved at 121°C for 1 h. After cooling to room temperature, the mixture was filtered through a medium-coarseness sintered glass crucible. The filtrate was transferred to a falcon tube, and the sugar concentrations measured by HPLC. Soluble lignin in the filtrate was determined by absorbance at 205 nm (Dence 1992). The insoluble Klason lignin remaining in the crucible was washed with deionised water and the crucible dried overnight at 100°C. The amount of Klason lignin was then determined gravimetrically after weighing the oven-dried crucible, taking into account the initial moisture content of the sample.

### 2.1.6 Ethanol analysis and 5-hydroxymethyl furfural and furfural analysis

Ethanol was analyzed using gas chromatography on a Hewlett Packard 5890 GC equipped with a Stabilwax-DA column (30 m, 0.35 mm ID) and helium carrier gas (20 mL/min). Injection and Flame Ionisation Detector FID temperatures were 90° C and 250° C, respectively. The oven was heated at 45° C for 6 min and increased to 230° C at a rate of 20° C/min, then held at 230° C for 10 min. Standards were prepared using 99% ethanol at a range of concentrations. All samples and standards received 0.3 g/L butanol (Fisher) as an internal standard. Furfural and 5-hydroxymethyl furfural (HMF) were analyzed on a Summit HPLC (Dionex) equipped with a Lichrospher RP18 reversed phase column (Varian Instruments, Walnut Creek, CA) and a PDA-100 detector set at 280 nm. Standards were prepared using HMF and furfural diluted in water. Catechol (BDH) was added as an internal standard to all samples and standards prior to their filtration through a 0.45 µm syringe filter (Chromatographic specialities, Brockville, ON). The injection volume was 25 µL. The column was heated to 60° C for the duration of the analysis and eluted at a flow rate of 0.5 mL/min with a ternary gradient of 7.4 mM phosphoric acid (eluent A), acetonitrile (eluent B), and a 4:3:3 volume/volume solution of 7.4 mM phosphoric acid, methanol (Fisher), and acetonitrile (eluent C). The elution profile consisted of a 20 min gradient of 95% eluent A and 5% eluent C transitioning to 50% eluent A and 50% eluent C. This was followed by 4 min

of gradient to 100% eluent C, a 1 min hold, then a 1 min transition to 100% eluent B. After holding for 1 min, there was a 1 min transition from 100% eluent B back to 95% eluent A and 5% eluent C, followed by 10 min of re-equilibration to end the run for a total time of 38 min.

### 2.1.7 Fermentation

The fermentation of the steam pre-treated material is described here. The fermentation of the ethanol pre-treated substrates is described in the corresponding section.

### 2.1.8 Separate hydrolysis and fermentation:

Washed solids were diluted to 5% (w/v) consistency with the WSF and the solution adjusted to pH 4.8 with 50% NaOH. The total volume used was 70 mL in order to allow enough liquid to be recovered for the fermentation step. Cellulase at 40 FPU/g glucan and β-glucosidase at 20 IU/g glucan were added and the flasks incubated in an orbital shaker at 50° C and 150 rpm for 72 h. Periodically, 400 µl samples were taken over 72 h, boiled for 5 min and stored at -20° C. After completion of the hydrolysis step, the reaction mixture was boiled for 5 min, and then centrifuged at 10,000 rpm for 10 min to separate the hydrolysate, which was removed and measured into clean flasks. The pH was adjusted to 6 with 50% NaOH and 5 g/L yeast added. The total volume for the fermentation was 40 mL. The flasks were incubated in an orbital shaker at 30° C and 150 rpm for a further 48 h. Periodically, 400 µL samples were taken, centrifuged at 10,000 rpm for 5 min and the supernatant stored at -20° C.

### 2.1.9 Simultaneous saccharification and fermentation:

Washed solids were diluted to 5% (w/v) consistency with the WSF and the solution adjusted to pH 5 with 50% NaOH. Nutrients (0.5 g/L $(NH_4)_2HPO_4$, 0.025 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L yeast extract and 1 g/L peptone) were added to each flask. Cellulase at 40 FPU/g glucan and β-glucosidase at 20 IU/g cellulose (Novozymes 188) were added and the flasks incubated in an orbital shaker at 50° C and 150 rpm for 6 h. After this prehydrolysis, 5 g/L yeast was added to each flask and the temperature reduced to 37° C. Periodically, 400 µL samples were taken, centrifuged at 10,000 rpm for 5 min, and the supernatant stored at –20° C.

# 3 Results and Discussion

## 3.1 Ethanol pre-treatment

Parameters such as cooking temperature, cooking time, catalyst [$H_2SO_4$] concentration, and ethanol concentration were varied and their response was examined. We used a Hartley small composite experimental design to maximize the potential to assess these trials. The original chemical composition of the lodgepole pine biomass used in these experiments is shown in Table 2. Condition selection was based on the results of preliminary experiments (data not shown)–see Table 3. The H-factor was derived from the cooking time and temperature. The H-factor is a common term used in pulp and paper which accounts for the time and temperature applied to a given lignocellulosic biomass sample (Vroom 1957). Sugar recovery and ease of hydrolysis of the resulting substrates at 2% (w/v) solids concentration were used as response variables to assess the efficiency of pre-treatment. A total of 21 points was generated for the small composite design (SCD), with 8 factorial points, 8 axial points, and 5 centre points. The value of α was 1.6818 given the $n_F$ value of 8. Data was analyzed using the Statistical Analysis System (SAS) V9 for Windows (SAS Institute Inc., Cary, NC). Of the conditions employed, the four representative conditions showed the greatest potential to maximize the sugar recovery and ease of hydrolysis from beetle-killed pine and those conditions were chosen and applied for treatment of healthy lodgepole pine.

**Table 2**. Chemical composition of untreated beetle-killed pine and healthy lodgepole pine wood chips.

| | Composition (% w/w oven-dried wood chips) | |
|---|---|---|
| Component | Beetle-killed | Healthy |
| Extractives | 4.7 | 3.4 |
| AIL | 26.0 | 26.2 |
| ASL | 0.3 | 0.4 |
| Arabinan | 1.4 | 1.9 |
| Galactan | 2.1 | 3.0 |
| Glucan | 47.6 | 44.9 |
| Xylan | 7.2 | 6.2 |
| Mannan | 12.4 | 11.5 |

[a] Abbreviations used: AIL–Acid-insoluble lignin; ASL–Acid-soluble lignin.

**Table 3**. Small composite design used to optimize EO pre-treatment of beetle-killed pine.

| | Coded | | | | Values | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Run | S | C | T | t | S, % | C, % | T, C | t, min | H-factor |
| LPP1 | -1 | 1 | -1 | -1 | 0.90 | 75 | 160 | 50 | 380 |
| LPP2 | -1 | 1 | 1 | -1 | 0.90 | 75 | 180 | 50 | 1957 |
| LPP3 | -1 | -1 | -1 | 1 | 0.90 | 55 | 160 | 70 | 512 |
| LPP4 | -1 | -1 | 1 | 1 | 0.90 | 55 | 180 | 70 | 2640 |
| LPP5 | 1 | -1 | -1 | -1 | 1.30 | 55 | 160 | 50 | 380 |
| LPP6 | 1 | -1 | 1 | -1 | 1.30 | 55 | 180 | 50 | 1957 |
| LPP7 | 1 | 1 | -1 | 1 | 1.30 | 75 | 160 | 70 | 512 |
| LPP8 | 1 | 1 | 1 | 1 | 1.30 | 75 | 180 | 70 | 2640 |
| LPP9 | 0 | 0 | -1.68 | 0 | 1.10 | 65 | 153 | 60 | 242 |
| LPP10 | 0 | 0 | 1.68 | 0 | 1.10 | 65 | 187 | 60 | 3954 |
| LPP11 | 0 | 0 | 0 | -1.68 | 1.10 | 65 | 170 | 43 | 770 |
| LPP12 | 0 | 0 | 0 | 1.68 | 1.10 | 65 | 170 | 77 | 1291 |
| LPP13 | -1.68 | 0 | 0 | 0 | 0.76 | 65 | 170 | 60 | 1031 |
| LPP14 | 1.68 | 0 | 0 | 0 | 1.44 | 65 | 170 | 60 | 1031 |
| LPP15 | 0 | -1.68 | 0 | 0 | 1.10 | 48 | 170 | 60 | 1031 |
| LPP16 | 0 | 1.68 | 0 | 0 | 1.10 | 82 | 170 | 60 | 1031 |
| LPP17 | 0 | 0 | 0 | 0 | 1.10 | 65 | 170 | 60 | 1031 |
| LPP18 | 0 | 0 | 0 | 0 | 1.10 | 65 | 170 | 60 | 1031 |
| LPP19 | 0 | 0 | 0 | 0 | 1.10 | 65 | 170 | 60 | 1031 |
| LPP20 | 0 | 0 | 0 | 0 | 1.10 | 65 | 170 | 60 | 1031 |
| LPP21 | 0 | 0 | 0 | 0 | 1.10 | 65 | 170 | 60 | 1031 |

LPP, lodgepole pine; T, cooking temperature (°C); t, time (min) at the cooking temperature; S, sulfuric acid (%, w/w oven-dried wood); C, ethanol concentration, (%, v/v); H-factor calculated from cook temperature and time determined by model.

Response surface methodology was used to evaluate the solids yield, glucose yield and the susceptibility to enzymatic hydrolysis of the resulting substrates. Four conditions from the design matrix were then selected to study the efficacy of the pre-treatment optimization for healthy lodgepole pine. Previous studies of beetle-killed wood found affected sapwood having lower lignin, carbohydrate and extractives content but increased permeability than sound sapwood(Wood and Layzell, 2003). The chemical composition of healthy and beetle-killed wood used in the research project was quite similar (Table 2). The most significant variations were the beetle-killed pine having a 3% higher glucan content and 1.3% higher extractives concentration than the healthy sample; however, this variation could also be due to experimental variation in the Klason measurement used for measuring the chemical composition of the

biomass. All other components fell within 1% deviation between the two wood samples. Due to the similarities in chemical compositions, it was expected that the healthy lodgepole pine would be similar to the beetle-killed pine in its responsiveness to the selected pre-treatment conditions.

**Table 4**. Centre-point pre-treated substrate composition and sugar yields from original material.

| Component | Composition (%) of Pre-treated Substrate | Recovery (%) from Original Material |
|---|---|---|
| AIL | 14.21 | 23.68 |
| ASL | 0.17 | 24.92 |
| Arabinan | 0.00 | 0.00 |
| Galactan | 0.00 | 0.00 |
| Glucan | 87.88 | 79.92 |
| Xylan | 0.79 | 4.75 |
| Mannan | 0.93 | 3.26 |

AIL–Acid-insoluble lignin; ASL–Acid-soluble lignin.

A larger batch of the substrate at the centre-point conditions (BK-CP, matrix conditions 17–21) was made by preparing 10 batches of pre-treated material (200 g untreated wood/batch) and mixing this larger batch prior to screening to remove pulping rejects. The total yield of solids was ~43% (w/w), of which 87.9% (w/w) was cellulose and approximately 14.4% (w/w) was lignin (Table 4). The substrate retained ~80% of the original cellulose from the untreated wood while the hemicellulose fraction was almost completely relegated to the liquid fraction during the pre-treatment. It was apparent that the H-factor played a significant role in the retention of hemicellulosic sugars in the solid fraction, as the shorter time and temperature resulted in more hemicellulosic sugars in the solid fraction (Table 5). A greater retention of hemicellulose was also accompanied by a lower *screened* solids yield (Table 5), since there was most likely a greater amount of *unpulped* reject material in the milder pre-treatment conditions such as beetle-killed pine P1 which became eliminated during the screening process.

**Table 5**. Chemical composition of substrates from EO pre-treatment of beetle-killed pine.

| Substrate | Solids Yield (%) | Composition (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Arabinan | Galactan | Glucan | Xylan | Mannan | Klason Lignin |
| BKP1 | 26.5 | 0.2±0.0 | 0.2±0.0 | 69.7±1.1 | 3.4±0.0 | 4.0±0.1 | 17.6 |
| BKP2 | 35.4 | 0.1±0.0 | 0.2±0.0 | 89.1±0.6 | 0.9±0.0 | 0.9±0.1 | 5.0 |
| BKP3 | 11.1 | 0.1±0.0 | 0.2±0.0 | 68.8±0.1 | 3.0±0.0 | 2.9±0.0 | 22.8 |
| BKP4 | 37.7 | 0.1±0.0 | 0.1±0.0 | 84.0±0.4 | 0.8±0.0 | 0.7±0.1 | 11.2 |
| BKP5 | 9.9 | 0.1±0.0 | 0.2±0.0 | 66.6±6.1 | 2.3±0.1 | 2.3±0.0 | 22.9 |
| BKP6 | 38.5 | 0.1±0.0 | 0.2±0.0 | 81.8±0.6 | 0.7±0.0 | 0.7±0.0 | 12.2 |
| BKP7 | 41.5 | 0.1±0.0 | 0.2±0.0 | 77.4±2.5 | 2.0±0.1 | 2.1±0.1 | 13.2 |
| BKP8 | 31.2 | 0.1±0.0 | 0.1±0.0 | 85.1±0.9 | 0.5±0.1 | 0.6±0.1 | 7.9 |
| BKP9 | 7.2 | 0.1±0.0 | 0.2±0.0 | 64.1±0.9 | 3.6±0.1 | 4.5±0.1 | 20.8 |
| BKP10 | 27.6 | 0.1±0.0 | 0.2±0.0 | 82.2±1.3 | 0.5±0.0 | 0.6±0.0 | 11.1 |
| BKP11 | 43.0 | 0.1±0.0 | 0.2±0.0 | 78.4±1.1 | 1.9±0.0 | 1.8±0.1 | 12.8 |
| BKP12 | 40.8 | 0.1±0.0 | 0.2±0.0 | 81.0±0.2 | 1.4±0.0 | 1.4±0.0 | 8.8 |
| BKP13 | 41.6 | 0.1±0.0 | 0.2±0.0 | 74.6±0.2 | 2.3±0.0 | 2.2±0.0 | 13.3 |
| BKP14 | 39.2 | 0.1±0.0 | 0.2±0.0 | 84.6±0.6 | 1.1±0.0 | 1.1±0.1 | 8.2 |
| BKP15 | 33.5 | 0.0±0.0 | 0.0±0.0 | 68.5±0.4 | 0.7±0.0 | 0.5±0.0 | 22.8 |
| BKP16 | 39.8 | 0.0±0.0 | 0.0±0.0 | 79.3±0.7 | 0.8±0.0 | 0.8±0.1 | 10.1 |
| BKP17 | 41.5 | 0.1±0.0 | 0.2±0.0 | 83.1±0.7 | 1.5±0.0 | 1.5±0.0 | 10.3 |
| BKP18 | 41.7 | 0.1±0.0 | 0.2±0.1 | 80.8±0.7 | 1.5±0.3 | 1.5±0.4 | 10.6 |
| BKP19 | 40.3 | 0.1±0.0 | 0.1±0.0 | 78.0±0.4 | 1.5±0.3 | 1.4±0.3 | 8.9 |
| BKP20 | 41.7 | 0.1±0.0 | 0.1±0.0 | 80.7±0.5 | 1.2±0.0 | 1.2±0.1 | 10.2 |
| BKP21 | 41.3 | 0.1±0.0 | 0.2±0.0 | 82.9±1.4 | 1.5±0.0 | 1.5±0.0 | 10.3 |
| Scale BK-CP | 41.3 | 0.1±0.0 | 0.2±0.0 | 82.0±0.5 | 1.3±0.1 | 1.3±0.2 | 10.1 |

BKP, Beetle-killed lodgepole pine; BK-CP, Beetle-killed pre-treated substrate centre-point

**Table 6**. Conversion of cellulose to glucose after enzymatic hydrolysis of substrates from EO pre-treatment of lodgepole pine.

| Substrate | Conversion (%) of cellulose to glucose | | | | | | | Conversion (%) of original hexose |
|---|---|---|---|---|---|---|---|---|
| | 0 hr | 1 hr | 3 hr | 6 hr | 12 hr | 24 hr | 48 hr | |
| BKP1 | 0 | 16.7 | 24.8 | 29.9 | 35.0 | 40.9 | 46.8 | 16.0 |
| BKP2 | 0 | 32.2 | 61.6 | 86.6 | 99.4 | 107.3 | 106.3 | 59.2 |
| BKP3 | 0 | 20.9 | 32.9 | 39.7 | 43.2 | 50.3 | 52.8 | 7.4 |
| BKP4 | 0 | 34.9 | 65.3 | 88.4 | 100.6 | 104.8 | 104.8 | 58.6 |
| BKP5 | 0 | 19.8 | 31.5 | 36.3 | 42.9 | 47.6 | 54.0 | 6.6 |
| BKP6 | 0 | 33.9 | 63.7 | 90.0 | 103.4 | 108.7 | 108.8 | 60.5 |
| BKP7 | 0 | 26.9 | 44.8 | 59.1 | 72.9 | 84.6 | 87.7 | 50.6 |
| BKP8 | 0 | 33.7 | 64.1 | 88.1 | 101.8 | 105.7 | 108.0 | 50.6 |
| BKP9 | 0 | 14.8 | 22.2 | 25.9 | 30.4 | 33.2 | 36.4 | 3.1 |
| BKP10 | 0 | 29.9 | 58.8 | 82.9 | 100.4 | 108.2 | 110.6 | 44.1 |
| BKP11 | 0 | 31.4 | 59.1 | 76.8 | 92.6 | 102.8 | 101.3 | 61.1 |
| BKP12 | 0 | 35.9 | 70.0 | 89.5 | 98.2 | 105.5 | 104.2 | 61.2 |
| BKP13 | 0 | 28.4 | 50.4 | 64.1 | 75.4 | 90.1 | 96.0 | 53.6 |
| BKP14 | 0 | 38.3 | 74.6 | 93.7 | 99.6 | 101.2 | 99.3 | 58.5 |
| BKP15 | 0 | 26.3 | 45.4 | 56.7 | 66.4 | 73.8 | 77.0 | 31.1 |
| BKP16 | 0 | 23.8 | 47.0 | 65.4 | 76.3 | 88.2 | 92.4 | 51.5 |
| BKP17 | 0 | 28.9 | 56.0 | 71.9 | 81.3 | 87.8 | 87.8 | 53.9 |
| BKP18 | 0 | 28.3 | 55.9 | 74.3 | 84.2 | 88.0 | 91.7 | 54.9 |
| BKP19 | 0 | 29.0 | 56.8 | 73.4 | 80.5 | 90.5 | 91.6 | 54.4 |
| BKP20 | 0 | 29.1 | 55.6 | 74.4 | 84.7 | 89.0 | 89.1 | 53.2 |
| BKP21 | 0 | 24.2 | 54.4 | 72.8 | 81.5 | 88.4 | 89.0 | 53.7 |
| LPP10 | 0 | 35.6 | 51.5 | 59.4 | 76.7 | 99.3 | 104.3 | 65.7 |
| LPP13 | 0 | 21.7 | 27.8 | 30.8 | 37.2 | 44.8 | 50.4 | 31.0 |
| LPP14 | 0 | 27.6 | 35.4 | 41.9 | 50.5 | 58.3 | 64.1 | 25.3 |
| LPPCP | 0 | 37.4 | 49.7 | 57.5 | 72.3 | 89.9 | 96.7 | 53.9 |

BKP, Beetle-killed lodgepole pine; LPP, Healthy lodgepole pine; LPPCP, healthy lodgepole pine pre-treated substrate centre-point

The extents of hydrolysis employing 10 FPU/g of cellulases supplemented with 20 IU/g cellulose of beta-glucosidase at 2% (w/v) solids showed that most substrates reached hydrolysis yields of at least 88% after 48 h, with five substrates having 100% cellulose conversion within 12 h (Table 6). The results of the surface response analysis (Figure 1 and 2) indicate that the temperature had a significant effect on the ability to obtain a substrate that was readily hydrolyzed by cellulases. Four representative conditions were chosen to be applied to compare their effects on beetle-killed pine versus healthy lodgepole pine, based on the conditions' ability to produce a readily hydrolyzed substrate while maximizing the retention of hexoses in the solid fraction, including the centre-point (170° C; 60 min; 1.10% $H_2SO_4$; 65% ethanol), beetle-killed pine P 10, 13 and 14 (Table 6). Although conversions between 90% and 100% were achieved, this reflected a 54%–60% recovery of hexoses in the untreated material.

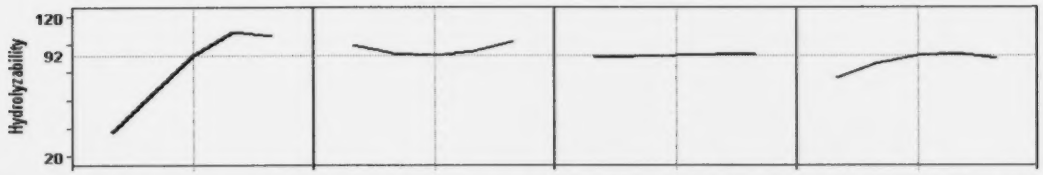
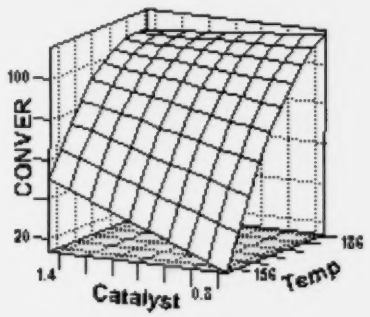



**Figure 1.** Effects of process variables on substrate hydrolyzability at 2% (w/v) solids, solids yield (% of original feedstock) and glucose yield (% of original glucan in feedstock) from EO pre-treatment of beetle-killed pine.




**Fixed levels:** **time = 60.000015** **Ethanol = 65.000015**

**Figure 2.** Response surface of temperature and catalyst concentration on substrate hydrolyzability at 2% (w/v) solids of EO pre-treatment of beetle-killed pine.

Comparing the application of conditions centre point (CP), 10, 13, and 14 to the beetle-killed pine versus healthy lodgepole pine revealed that although the wood chemical compositions were similar (Table 7), the healthy lodgepole pine responded differently. Previous studies of beetle-killed wood found affected sapwood to have lower lignin, carbohydrate and extractives content but more permeability than sound sapwood. Therefore, it is possible that the increased permeability of the beetle-killed pine wood enhanced the penetration of the pre-treatment liquor into the wood chips during the pulping process. In papermaking, chips are frequently pre-steamed prior to exposure to the pulping liquor in order to improve permeability due to the expulsion of air from the chip pores by the water vapour. The next set of experiments was aimed at evaluating the fermentability of the hydrolyzate resulting from the enzymatic hydrolysis of organosolv pre-treated substrates.

**Table 7**. Chemical composition of corresponding substrates from organosolv pre-treatment of beetle-killed pine and healthy lodgepole pine.

| | CP | | 10 | | 13 | | 14 | |
|---|---|---|---|---|---|---|---|---|
| Component | BK | H | BK | H | BK | H | BK | H |
| Solids Yield | 41.3 | 45.8 | 27.6 | 31.3 | 41.6 | 47.2 | 39.2 | 47.4 |
| AIL | 10.1 | 15.1±0.0 | 11.1 | 15.9±0.0 | 13.3 | 16.1±0.0 | 8.2 | 18.1±0.0 |
| Glucan | 81.1±0.7 | 79.0±0.6 | 82.2±1.3 | 79.6±2.6 | 74.6±0.2 | 74.9±2.8 | 84.6±0.6 | 70.2±2.6 |
| Arabinan | 0.1±0.0 | 0.0±0.0 | 0.1±0.0 | 0.0±0.0 | 0.1±0.0 | 0.0±0.0 | 0.1±0.0 | 0.0±0.0 |
| Galactan | 0.2±0.0 | 0.0±0.0 | 0.2±0.0 | 0.0±0.0 | 0.2±0.0 | 0.0±0.00 | 0.2±0.0 | 0.0±0.0 |
| Xylan | 1.4±0.1 | 0.9±0.0 | 0.5±0.0 | 0.8±1.1 | 2.3±0.0 | 2.2±0.2 | 1.1±0.0 | 1.5±0.1 |
| Mannan | 1.4±0.2 | 0.8±0.1 | 0.6±0.0 | 0.8±0.9 | 2.2±0.0 | 1.8±0.1 | 1.1±0.1 | 1.3±0.1 |

As mentioned in the previous sections, it has been suggested that increasing the concentration of ethanol from fermentation can be accomplished by increasing the sugar concentrations of the feed stream (Mohagheghi et al. 1992). Various strategies have been developed to achieve high conversions of cellulosic slurries approaching or exceeding 10% (w/v) solids concentration with the goal of improving ethanol concentrations. Simultaneous saccharification and fermentation has been used to improve the processing of higher solids loadings (> 10%) and improve ethanol yields (Varga et al. 2004). It was originally hypothesized that as cellulase enzymes liberate soluble glucose, yeasts can simultaneously ferment the sugars so as to decrease the end-product inhibition toward the enzyme and thus increase hydrolysis efficiency. Another approach to improve processing at elevated solids is to pre-incubate the enzyme–substrate mixture hybrid hydrolysis and fermentation (HHF), allowing for liquefaction of the substrate and facilitating more homogeneous distribution of all the reaction components (Hodge 2005). Supernatants from the hydrolysis of BK-CP conditions using 20 mg/g cellulose of cellulase without β-glucosidase supplementation were fermented to complete SHF processing. Yields from SSF and SHF processing were evaluated to investigate whether reducing end-product inhibition can increase ethanol production. For the SHF fermentation, after hydrolysis of the substrate at an enzyme loading of 20 mg protein $g^{-1}$ cellulose, the flasks were placed in a boiling water bath for 5 min and the reaction mixture was then centrifuged at 10,000 rpm for 10 min. The supernatant was transferred into clean flasks and the pH was adjusted to 6 with 50% sodium hydroxide. Yeast was added to make a total reaction volume 50 mL and a final yeast concentration of 3 g $L^{-1}$. The flasks were incubated at 30°C with constant agitation at 150 rpm for 24 h. Similarly, for the SSF, cellulases were loaded at 20 mg protein $g^{-1}$ and cellulose and yeast were added simultaneously to the reaction mixtures which were suspended at 10% or 15% (w/v) solid substrate before being incubated at 37° C for 48 h with constant agitation at 150 rpm in a rotary shaker. No supplemental β-glucosidase was used and tetracycline was added to a final concentration of 40 μg $mL^{-1}$ to minimize bacterial contamination. For HHF processing, cellulase again was loaded at 20 mg protein $g^{-1}$ cellulose and was added to reaction mixtures of 10% or 15% (w/v) solid substrate and incubated at 50° C for 12 h with constant agitation at 150 rpm in a rotary shaker. No supplemental β-glucosidase was used and tetracycline was added to a final concentration of 40 μg $mL^{-1}$ to minimize bacterial contamination. At 12 h, the flasks were cooled to 37° C before the addition of yeast to the reaction mixture, and then incubated for 36 h at 37° C with constant agitation at 150 rpm in a rotary shaker. Total reaction time including both temperatures was 48 h.

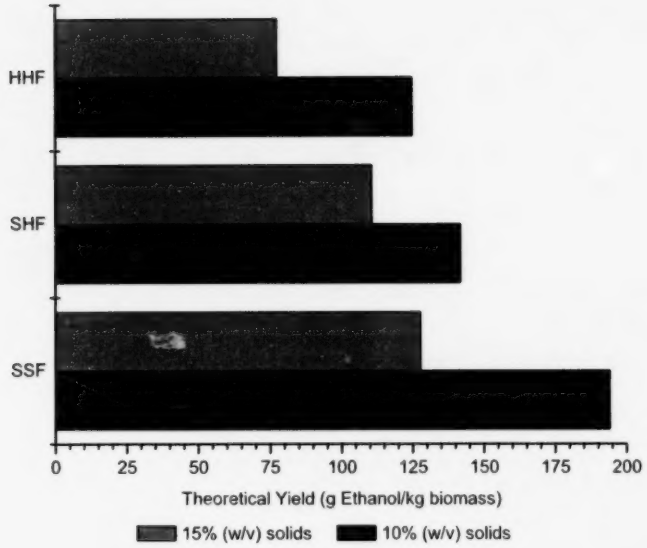



**Figure 3.** Fermentation results in SHF, SSF and HHF configurations for organosolv pre-treated beetle-killed pine at the centre-point conditions.

It was apparent from the data that the fermentation of the BK-CP substrate was compromised when the solids loading was increased from only 10% to 15% solids (Figure 3). As mentioned previously, performing the hydrolysis reactions at the flask scale at elevated solids loadings has been shown to decrease cellulose conversion (Xiao et al. 2004). The use of SSF was more beneficial at the 10% solids loading compared to the SHF, potentially because of the decreased end product inhibition due to the decreased amount of β-glucosisdiase added to the reactions for fermentation. Conversion during SHF processing indicated that it was possible to obtain an ethanol concentration of 31 g/L and a yield of 124 g of ethanol per kg of biomass which is approximately a 76% conversion of the initial glucose in the wood.

## 3.2 Comparing the hydrolysis of ethanol organosolv pre-treated lodgepole pine to Douglas-fir

Historically, it has been shown that the pre-treatment of softwoods to hydrolyze at minimal enzyme loadings has been challenging, as many studies have examined the application of cellulases loadings in the 20 FPU/g cellulose range (Boussaid et al. 2000; Yang et al. 2002), at solids loadings of 1-5% (w/v). Furthermore, the species Douglas-fir has been shown to be particularly recalcitrant when applying steam pre-treatment to improve enzymatic hydrolysis. However, recent work has shown that the organosolv process has the potential to be applied to softwood substrates (Pan et al. 2006) and may be more robust in its ability to process softwood biomass. Therefore, conditions for ethanol pre-treatment were applied to both healthy and beetle-killed pine in addition to Douglas-fir. The severity of the organosolv pre-treatment was selected for *optimum* performance, defined as the highest recovery of cellulose and the most effective delignification. Based on our experiences with hardwood chips using EO for delignification, the following conditions were selected: 180°C, 50 min soak time, 0.9% w/v $H_2SO_4$ catalyst, a 75% ethanol v/v in the pre-treatment liquor, 7:1 liquor ratio, and 200 g oven-dry weight for each sample loaded into the digester system at the University of British Columbia Process Development Unit Laboratory digester system.

**Table 8**. Percentage of pulp yield after EO pre-treatment of healthy lodgepole pine, beetle-killed pine and Douglas-fir samples.

| | healthy lodgepole pine | beetle-killed pine | Douglas-fir |
|---|---|---|---|
| Pulp yield (wood chips including rejects) | 44 % | 39 % | 37 % |
| Pulp yield (wood chips after screening) | 43 % | 39 % | 37 % |
| Reject in pulp | 2.2 % | 1.4 % | 1.2 % |
| Pulp yield (after screening without rejects) | 97 % | 98 % | 98 % |

It was apparent from the results that the substrates possessed a relatively low percentage of reject material in a range of less than 2.3% for all three samples. Similar to our previous observations, the healthy lodgepole pine resulted in higher solids yields during pre-treatment. Therefore, the percentage of substrate fiber that passed through the screen was as high as approximately 98% for Douglas-fir since it was apparent that the wood was thoroughly fiberized (Table 8). It was also quite interesting that the Douglas-fir wood, which in previous experience in our Forest Products Biotechnology Group has been known for its recalcitrance to pre-treatment and hydrolysis, responded in a similar fashion to the healthy lodgepole pine and beetle-killed pine. As performed in the previous section, additional pre-treatment conditions for optimization may be performed to further increase pulp yield, including increasing sulfuric acid concentration, lowering the liquor to solid ratio, and extending soak time depending on the parameter. The liquid streams from the EO pre-treatment were collected for oligosaccharide-monosaccharide analysis, lignin content and determination of fermentation inhibitory compounds. The liquid stream is typically composed of ethanol, lignin, hemicellulose and fermentation inhibitors such as furfural and HMF generated from the degradation of sugars. As hexoses and pentose are dehydrated during the high pressure and high temperature treatment in EO, they can generate furfural and HMF, respectively, which are regarded as fermentation inhibitors (Klinke et al. 2004)and can potentially inhibit downstream fermentation processes. Previous literature has suggested that glucose fermentations are severely affected with furfural and HMF at concentrations of 0.30-0.46 g/L or higher (Parajó et al. 1996). Due to the potential negative effect of these furan compounds on the efficiency of subsequent fermentation, an inhibitor analysis was performed to monitor both furfural and HMF concentrations in the liquid stream after pre-treatment. The results indicated that the concentration of furfural and HMF in the liquid stream was relatively low (Table 9). Further analysis of the liquid stream indicated the presence of significant amounts of oligomeric polysaccharides, likely originating from the acidic breakdown of the hemicellulose component in the wood during the approximate 2 h heating time of the organosolv pre-treatment process at the conditions applied here.

**Table 9**. Concentration of furfural and HMF in the liquid stream.

| | [Furfural] g/L (stdev) | [HMF] g/L (stdev) |
|---|---|---|
| Healthy lodgepole pine | 0.10 (0.0035) | 0.14 (0.005) |
| Beetle-killed lodgepole pine | 0.14 (0.0036) | 0.14 (0.001) |
| Douglas-fir | 0.11 (0.0124) | 0.15 (0.003) |

More than 50% of the arabinose, galactose, xylose and mannose from the wood chips were solubilized into the liquid stream during the ethanol pre-treatment process (Tables 10 and 11). This was expected as hemicelluloses are branched and possess less polymerization than linear cellulose molecules, and are thus more labile (Sjöström 1993). Overall, a significant amount of the initial glucose was retained in the solid fraction of the substrate.

**Table 10**. Water-soluble monosaccharides (in g/L) in the liquid stream of organosolv pre-treatment.

| Sample | Arabinose | Galactose | Glucose | Xylose | Mannose |
|---|---|---|---|---|---|
| Healthy lodgepole pine | 0.7 | 1.1 | 1.8 | 2.5 | 3.7 |
| BK-lodgepole pine | 0.6 | 1.1 | 2.5 | 2.5 | 3.1 |
| Douglas-fir | 0.4 | 1.0 | 3.0 | 1.6 | 3.5 |

**Table 11**. Water-soluble oligosaccharides (in g/L) in the liquid stream after organosolv pre-treatment.

| Sample | Arabinose | Galactose | Glucose | Xylose | Mannose |
|---|---|---|---|---|---|
| Healthy lodgepole pine | 1.5 | 2.7 | 4.0 | 5.2 | 11.7 |
| Beetle-killed pine | 1.4 | 3.0 | 5.8 | 5.1 | 10.4 |
| Douglas-fir | 0.8 | 2.4 | 6.3 | 3.3 | 10.6 |

Chemical analysis of the substrate after the pre-treatment of healthy lodgepole pine (87.2 g of content per 100 g sample), beetle-killed pine (88.6 g of content per 100 g sample) and Douglas-fir (90.3 g of content per 100 g sample) indicated that maximum glucan was recovered in the substrate derived from Douglas-fir samples. The higher glucan content of the pre-treated solid fraction does not necessarily represent higher recovery of glucose. We need to also consider the pulp yield and the glucose in the liquid fraction. This was surprising, considering the apparent recalcitrance of the Douglas-fir to steam pre-treatment observed in our previous studies. The organosolv pre-treatment seemed to be more effective in processing the Douglas-fir wood compared to the steam pre-treatment process possibly due to the removal of lignin which occurs during the EO process. Similarly to our previous observations, maximum xylan (1.5 g/100 g of sample) and mannan (1.5 g/100 g of sample) content, in addition to the higher lignin content, was observed with the pre-treated substrate from the healthy lodgepole pine. Considering the overall similarity among the three pre-treated softwood samples with respect to chemical composition, it was of great interest to assess their susceptibility to enzymatic hydrolysis.

**Table 12.** Chemical composition of organosolv pre-treated healthy lodgepole pine, beetle-killed pine and Douglas-fir samples.

| | Arabinan* | Galactan | Glucan | Xylan | Mannan | AIL | ASL | Ash |
|---|---|---|---|---|---|---|---|---|
| healthy lodgepole pine | 0.1 | 0.1 | 87.2 | 1.5 | 1.5 | 18 | 0.3 | 0.1 |
| beetle-killed pine | 0.1 | 0.1 | 88.6 | 0.9 | 1.0 | 17.6 | 0.3 | 0.1 |
| Douglas-fir | 0.1 | 0.1 | 90.3 | 0.6 | 1.0 | 18.1 | 0.3 | 0.1 |

*g of content per 100 g sample. Abbreviations used: AIL–Acid-insoluble lignin; ASL–Acid-soluble lignin.

Enzymatic hydrolysis of the cellulose component was complete within 48 h regardless of decreasing enzyme loading and increasing solids loading during enzymatic hydrolysis (Figure 4). For all three substrates, higher enzyme loading resulted in a higher initial rate of hydrolysis, especially within the first 12 h, suggesting that the enzyme loading only affected the initial rate of hydrolysis but not the overall sugar conversion. Similarly, when increasing the solids loading to 5% w/v, the hydrolysis reaction tended to initially slow at the lower enzyme loading but recovered to achieve a hydrolysis loading of 100%.

The reaction mechanism of cellulase has been hypothesized to involve the endo-1,4-β-glucanases randomly cleaving β-1-4 glycosidic linkages over the surface, while exo-1,4-β-glucanases cleave glucose or cellobiose from both reducing and non-reducing ends (Zhang and Lynd 2004). It has been hypothesized that this reaction mechanism is particularly limited to the surface area of the cellulose fibre exposed; due to the size and structure of the enzyme, it is impossible to penetrate the surface and access the cellulose chain in the centre of bundled fibre structure (Mansfield et al. 1999). The initial slowdown in the rate of hydrolysis at the elevated solids loading, in this case, may have been mitigated at the later stages of the reaction by the liquefaction of the reaction mixture over the duration of the reaction. This may have improved the mixing and accessibility of the substrate to the enzyme. The hydrolysis rate decreased after 12 h, which may be due to end-product inhibition, since glucose and cellobiose produced during hydrolysis can inhibit cellulase enzymes (Todorovic et al. 1987). Alternatively, the rate of enzymatic hydrolysis may have decreased due to the decrease in accessible cellulose, since the proportion of lignin in the substrate also increases as the cellulose is hydrolyzed during the reaction. It has been shown during our previous studies that the presence of lignin in the substrate is detrimental to the action of cellulolytic enzymes (Berlin et al. 2006).

One of the most interesting results observed during this study was that the initial rate of enzymatic hydrolysis of the beetle-killed pine was higher than that of the healthy lodgepole pine, even at low enzyme loading of 10 FPU/g glucan (Figure 4, 5, and 6). The extensive destruction to the chemical structure of beetle-killed pine may have devastated the economical value of these timbers for structural applications; however, the increased receptivity of the beetle-killed pine to pre-treatment and enzymatic hydrolysis may be beneficial for applying beetle-killed pine wood for bioconversion to ethanol in processes utilizing EO pre-treatment.

A key difference between the feedstocks is that beetle-killed pine samples were cut from standing dead trees, while healthy samples were still alive when harvested. It has also been reported that the sapwood portion of beetle-killed pine has increased permeability which may have in this case facilitated the penetration of the pre-treatment liquor into the chips and improved delignification during orgnanosolv pre-treatment (Woo et al. 2005).

Another result of increased drying is the degradation of wood fibers and an increase in fines (Koch 1996; Thomas 1986). This may have allowed the wood to be more easily broken down during the pre-treatment. Other possible factors which could possibly improve the bioconversion of beetle-killed pine could be due to colonizing microorganisms. By comparing beetle-killed to healthy wood, it is apparent that the lignin and extractive concentrations are similar (Table 2). Lieu et al. (1979) also found that there was no significant difference in the chemical composition of healthy and standing dead lodgepole pine. It is possible that degradation of hemicellulose to monomers could increase the accessibility of the cellulose while the amount of sugars remained constant and thus could also possibly improve access to cellulase enzymes. Another possibility is modification of the wood fibers by sapstaining fungi or other organisms. Fleet et al. (2001) showed that although sapstaining fungi consume small amounts of mannose and glucose, their primary energy source is lipids. Alternatively, the differences in behaviour between the healthy and beetle-killed wood during pre-treatment may be due to the differences in specific gravity and permeability observed between the beetle-killed pine and sound lodgepole pine (Woo et al. 2005).

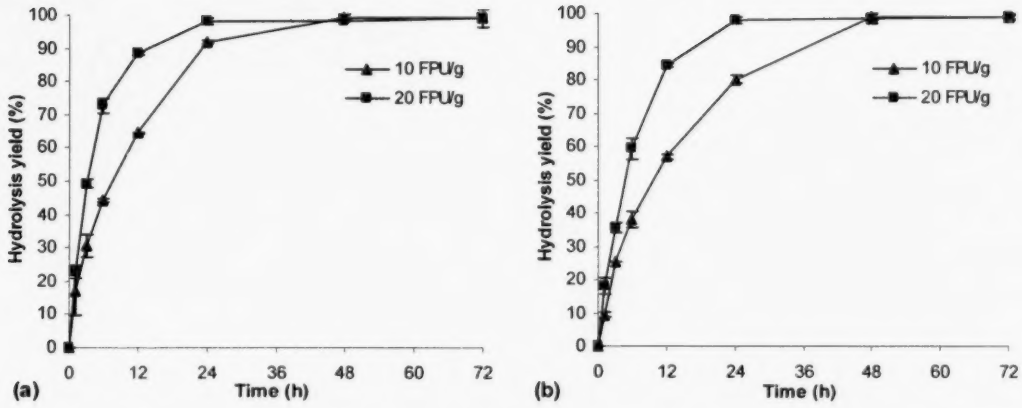



**Figure 4.** Enzymatic hydrolysis of organosolv pre-treated healthy lodgepole pine at different cellulase loading (10 and 20 FPU/g of glucan) using 2% (a) and 5% (b) pulp consistencies.

**Note:** Conditions used: temperature, 50°C; pH, 4.8; shaking speed, 150 rpm; cellulase to β-glucosidase ratio, 1:2.

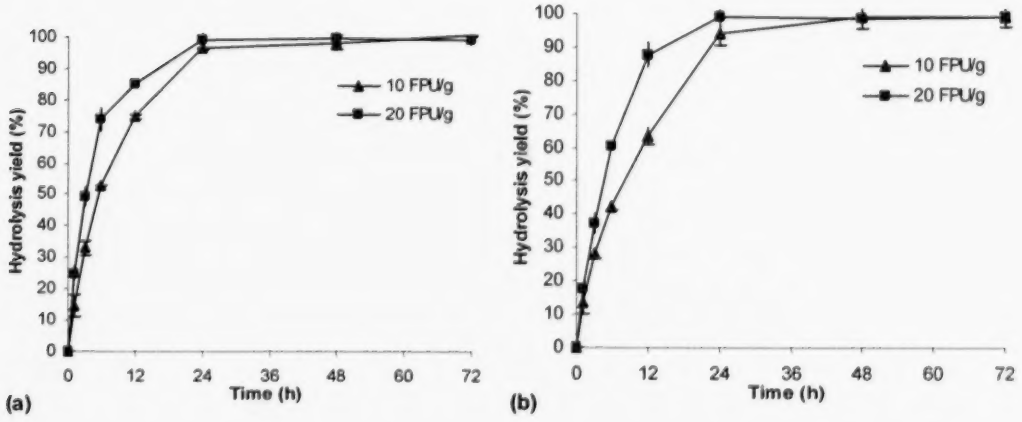



**Figure 5.** Enzymatic hydrolysis of organosolv pre-treated beetle-killed pine at different cellulase loading (10 and 20 FPU/g of glucan) using 2% (a) and 5% (b) pulp consistencies.

**Note:** Conditions used: temperature, 50° C; pH, 4.8; shaking speed, 150 rpm; cellulase to β-glucosidase ratio, 1:2.

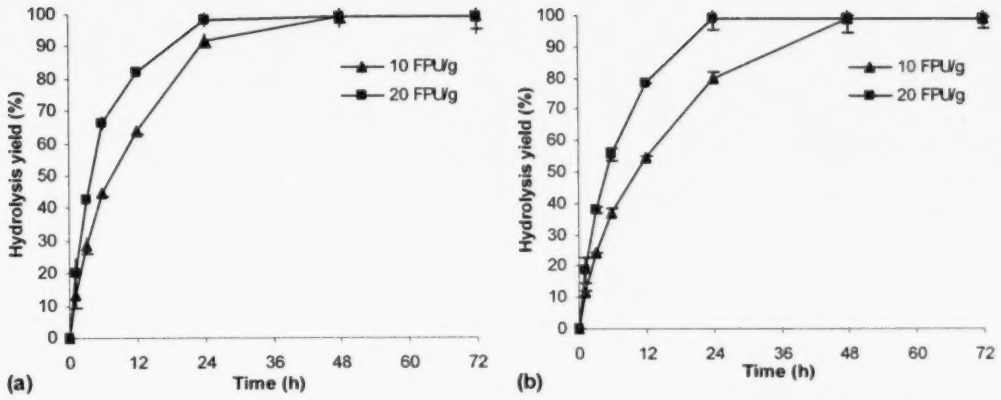



**Figure 6.** Enzymatic hydrolysis of organosolv pre-treated Douglas-fir at different cellulase loadings (10 and 20 FPU/g of glucan) using 2% (a) and 5% (b) pulp consistencies.

**Note:** Conditions used: temperature, 50°C; pH, 4.8; shaking speed, 150 rpm; cellulase to β-glucosidase ratio, 1:2.

## 3.3 Organosolv pre-treatment while varying the liquor-to-wood ratio

All our studies to this point have used a liquor-to-wood ratio of 7:1 (liquor volume:wood chip mass). This ratio was employed to improve lignin solubilization while still allowing enough space in the pulping digester at the Process Development Unit Laboratory for suspended wood chips to tumble and mix in the digester vessel using the rotating mixing method. But using a large volume of liquor may present challenges to recovery of sugars, sugar breakdown products, and ethanol re-used or employed as building blocks for other bioproducts. It was of interest to determine whether the recovery of sugars in the liquid stream at less diluted concentrations could be facilitated and whether the pre-treatment severity could be increased to produce substrates more amenable to enzymatic hydrolysis through altering the liquor-to-wood ratio in the pulping liquor. Typical kraft pulping is performed at a liquor:wood ratio of 3-4 (Smook 1992). Higher liquor:wood ratios can increase the degree of polymerization of pulp carbohydrates and slow the pulping reaction (Mortimer 1989). At the centre-point EO pre-treatment conditions (170°C, 1.1% $H_2SO_4$, 50 min, 7:1 liquor wood ratio) the liquor-to-wood ratio was varied from 5:1 to 3:1 to 2:1. The acid catalyst concentration was kept constant in the liquor so as to ensure that the acid concentration would not be suddenly increased in the liquor by decreasing the liquor:wood ratio if the charge on wood were to be kept constant. For example, 1.1% $H_2SO_4$ on 200 g oven-dried wood chips at a liquor:wood ratio 7:1 would result in a lower $H_2SO_4$ concentration than if it were employed at a liquor-to-wood ratio of 3:1. Samples were also washed with water instead of ethanol after pre-treatment. All pre-treatments were performed on beetle-killed pine wood chips as described in previous sections.

The data indicated that the decrease in the liquor:wood ratio increased the pulping rejects. In fact, at the lowest liquor-to-wood ratio (2:1), the substrate did not receive adequate agitation within the digester system and was severely cooked. Furthermore, analysis of the liquid stream revealed that although the liquor volume was decreased significantly, the sugar concentrations in the liquid stream from pre-treatment were not drastically different, with the exception of the substrate prepared at the 3:1 ratio.

**Table 13**. The composition of wood after organosolv pre-treatment while varying the liquor-to-wood ratio at the 170° C, 50 min cook time and constant acid catalyst concentrations.

| | LW1 | LW2 | LW3 | LW4 |
|---|---|---|---|---|
| Liquor wood ratio | 7:1 | 5:1 | 3:1 | 2:1 |
| Solids recovery (%) | 43 | 51 | 49 | 47 |
| Rejects (%) | 1 | 2 | 15 | 31 |
| Arabinose (%) | 0 | 0.1 | 0.1 | 0.1 |
| Galactose (%) | 0 | 0.4 | 0.2 | 0.3 |
| Glucose (%) | 94 | 79 | 68 | 60 |
| Xylose (%) | 0 | 1.5 | 3.3 | 4.0 |
| Mannose (%) | 0.2 | 1.2 | 3.3 | 5.1 |
| AIL (%) | 21 | 19 | 23 | 26 |

**Table 14**. Total (oligomeric and monomeric) sugar concentration in the liquid stream from the ethanol pre-treatment of beetle-killed pine at decreasing liquor-to-wood ratios in the University of British Columbia digester.

| | Sugar concentration (mg/mL) | | | | |
|---|---|---|---|---|---|
| Liquor-to-wood ratio | Ara | Gal | Glu | Xyl | Man |
| 7:1 | 2.4 | 5.8 | 20.2 | 9.7 | 16.7 |
| 5:1 | 2.1 | 5.4 | 14.3 | 9.3 | 15.5 |
| 3:1 | 4.7 | 9.1 | 9.0 | 14.0 | 25.0 |
| 2:1 | 1.6 | 3.4 | 3.3 | 4.2 | 7.7 |

Decreasing the liquor-to-wood ratio in the digesters not only increased the amount of pulping rejects particles but also compromised the enzymatic hydrolysis yields, which may be due to the increased lignin and/or hemicellulose content of the resulting solid substrates (Figure 7).

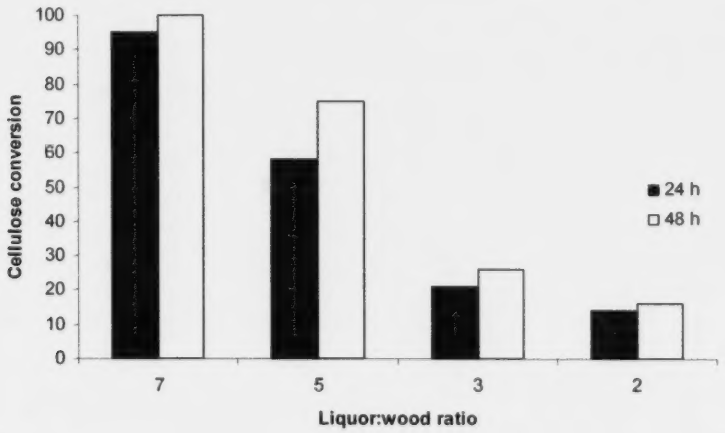



**Figure 7.** The effect of varying the liquor-to-wood ratio during the ethanol pre-treatment of beetle-killed pine.

**Note:** The hydrolysis reaction for the substrate prepared at the 7:1 liquor:wood ratio was performed at a 5% w/v solids loading while hydrolysis of the other substrates was performed at the 2% solids loading.

## 3.4 Steam pre-treatment

Typically, pre-treatment optimization involves a complete response surface methodology study to find the optimum levels of multiple variables. This type of modeling works well on homogenous, consistent substrates. For lodgepole pine, the large amount of variability inherent in trees from an area as large and diverse as British Columbia would require an extremely large sample size to be representative. Even with a large sample size, the effects of the beetle will change the characteristics of trees over time. For these reasons we chose to select the *optimum* pre-treatment conditions that provided the best overall hexose recovery (hemicellulose and cellulose derived), cellulose hydrolysability and overall ethanol yield from a range of seven conditions.

The seven conditions are detailed in Table 15, with the least severe (S1) being the optimized conditions used to pre-treat Douglas-fir (Boussaid et al. 2000) and the most severe (S7) being the conditions commonly used for spruce (Stenberg et al. 2000). The other five conditions were compromise conditions between these two extremes. Severity is measured using the severity factor Ro, which is calculated as $Ro = te^{(T-100)/14.75}$ where t is time and T is temperature (Overend et al. 1987).

**Table 15**. Conditions and corresponding severities used in optimizing pre-treatment of beetle-killed pine and resulting hexose recoveries.

| Severity Code | Temp (°C) | Time (min) | $SO_2$ (% d.w.) | Severity ($logR_o$) |
|---|---|---|---|---|
| S1 | 195 | 4.5 | 4.5 | 3.45 |
| **S2** | **195** | **7.5** | **4** | **3.67** |
| **S3** | **200** | **5** | **4** | **3.64** |
| S4 | 205 | 5 | 2 | 3.79 |
| S5 | 200 | 10 | 2 | 3.94 |
| S6 | 210 | 5 | 2 | 3.94 |
| **S7** | **215** | **5** | **4.5** | **4.09** |

Substrates pre-treated at all seven conditions were initially evaluated for the hydrolysability of the solid fraction and fermentability of the water-soluble stream. Three of the seven, representing low, medium and high severity were subsequently chosen and characterized to determine their monomeric and oligomeric sugars, and HMF and furfural concentrations. The three substrates were then subjected to SSF and SHF and the overall ethanol yields compared in order to select the *optimum* pre-treatment condition (Figure 8).

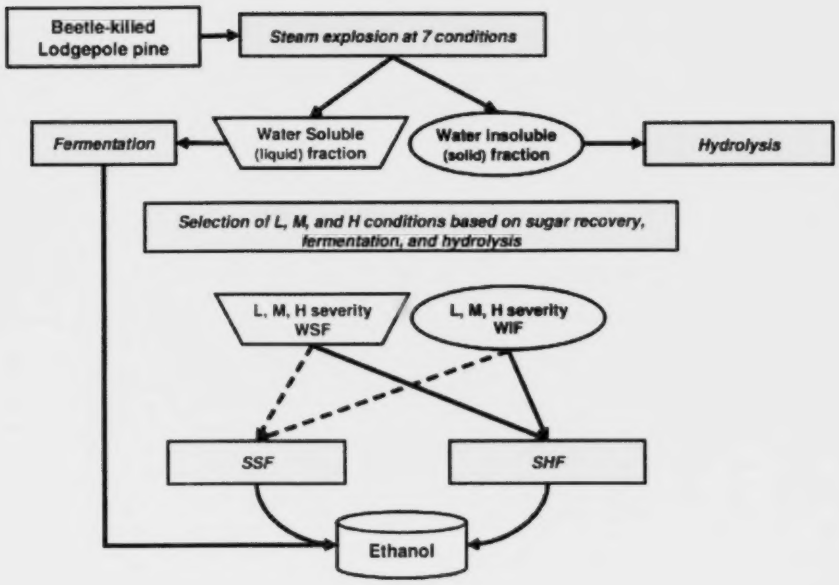



**Figure 8.** Pre-treatment optimization of beetle-killed pine.

The total recovery of sugars was derived by adding the amount of sugar found in the water-soluble and insoluble streams (after pre-treatment) and the liquids recovered from washing the steam gun and from washing the WIF. As expected, the recovery was lower at higher severity pre-treatments (Table 16).

**Table 16.** Sugar recovery after SO2-catalyzed steam pre-treatment of beetle-killed pine at 7 severities.

| | Sugar recovery (% of original sugars) | | | | |
|---|---|---|---|---|---|
| Severity | Arabinose | Galactose | Glucose | Mannose | Xylose |
| S1 | 83 | 85 | 100 | 75 | 70 |
| **S2 (Low)** | **84** | **99** | **100** | **90** | **85** |
| **S3 (Medium)** | **78** | **94** | **100** | **81** | **73** |
| S4 | 66 | 79 | 105 | 70 | 61 |
| S5 | 58 | 70 | 99 | 60 | 55 |
| S6 | 53 | 65 | 100 | 57 | 48 |
| **S7 (High)** | **50** | **69** | **97** | **53** | **41** |

Figure 9 shows the relationship between total hexose (glucose, mannose, and galactose) and pentose (arabinose and xylose) recovery and the logarithm of the severity factor $R_o$. $R_o$ correlates well with the amount of sugar recovered ($R^2$ = 0.74 for pentoses, and 0.96 for hexoses) even though the acid concentration is not included. The pentose recovery curve is much steeper than the hexose curve, showing the increased susceptibility of hemicellulosic sugars to degradation under severe conditions (Wu et al. 1999; Boussaid et al. 2000). Although the yeast *Saccharomyces cerevisiae* cannot ferment pentoses, minimizing their degradation is still important as degradation products such as furfural can inhibit the yeast during fermentation (Taherzadeh et al. 1997).

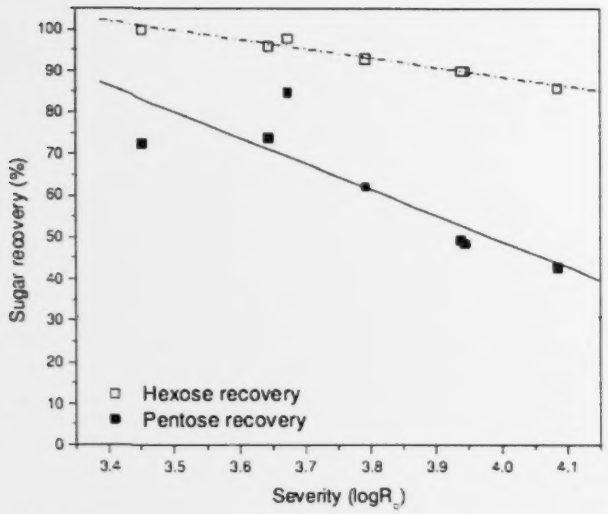



**Figure 9.** Relationship of pre-treatment severity to hexose and pentose recovery after $SO_2$-catalyzed steam pre-treatment of beetle-killed pine in 7 conditions.

Hydrolyzing the water-washed insoluble fraction at 2% consistency revealed that even the lowest severity pre-treatments (S1 and S2) produced substrates from which 69%–73% of the cellulose was hydrolysed to glucose after 72 h (Figure 10). The highest conversion, 96%, was from the substrate pre-treated at the highest severity (S7), clearly showing the relationship between pre-treatment severity and hydrolytic performance.

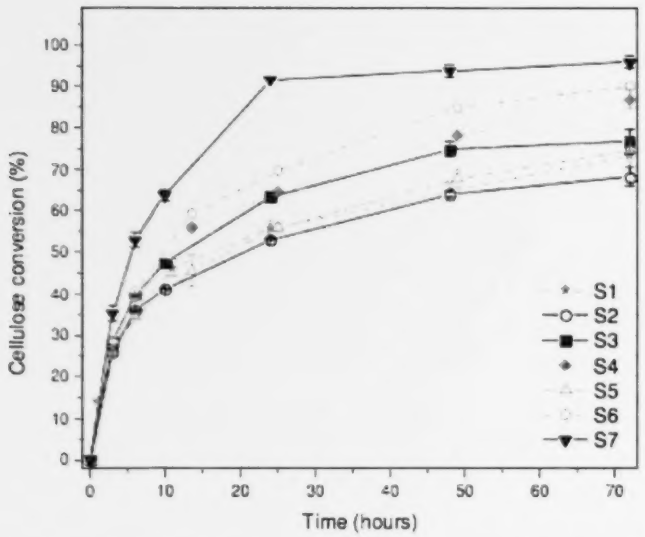



**Figure 10.** Conversion of cellulose to glucose after hydrolysis at 2% consistency of the WSF of beetle-killed pine pre-treated under 7 conditions.

As is normal after steam pre-treatment, the WIF of the seven substrates after pre-treatment contained primarily glucose and insoluble lignin (Table 17). Typically, as severity increases, cellulose decreases as it gets hydrolysed, and the resulting soluble glucose is separated into the WSF. This consequently increases the ratio of lignin to glucose in the pre-treated material.

**Table 17**. Composition of the washed, WIF of beetle-killed pine pre-treated at 7 severities.

| Severity | | g/100g dry weight | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Code | $\log R_o$ | Arabinose | Galactose | Glucose | Mannose | Xylose | AIL[a] | ASL[b] |
| S1 | 3.45 | 0.1 (0.0) | 0.1 (0.0) | 58.4 (0.8) | 0.5 (0.1) | 0.3 (0.0) | 43.9 (0.7) | 0.7 (0.0) |
| **S2 (Low)** | **3.67** | **0.1** (0.0) | **0.0** (0.0) | **61.4** (0.5) | **0.7** (0.1) | **0.4** (0.1) | **44.9** (0.1) | **0.6** (0.0) |
| **S3 (Medium)** | **3.64** | **0.1** (0.0) | **0.1** (0.0) | **59.6** (0.4) | **0.8** (0.2) | **0.2** (0.1) | **46.2** (0.2) | **0.7** (0.0) |
| S4 | 3.79 | 0.0 (0.0) | 0.0 (0.0) | 53.6 (0.6) | 0.2 (0.0) | 0.2 (0.0) | 44.4 (0.3) | 0.7 (0.0) |
| S5 | 3.94 | 0.0 (0.0) | 0.0 (0.0) | 56.8 (0.0) | 0.2 (0.2) | 0.2 (0.2) | 40.3 (0.8) | 0.6 (0.1) |
| S6 | 3.94 | 0.0 (0.0) | 0.0 (0.0) | 54.2 (0.3) | 0.3 (0.2) | 0.1 (0.2) | 44.9 (0.4) | 0.8 (0.1) |
| **S7 (High)** | **4.09** | **0.0** (0.0) | **0.0** (0.0) | **46.5** (0.4) | **0.0** (0.0) | **0.0** (0.0) | **58.1** (0.3) | **1.0** (0.0) |

[a]**Acid insoluble (Klason) lignin**
[b]**Acid soluble lignin**

**Note:** Values in parentheses indicate deviation from the mean.

The more severe the pre-treatment, the more lignin is removed from the surface of the cellulose, increasing the accessibility for cellulases. Figure 11 shows the relationship between the severity factor, log $R_o$, and hydrolytic conversion. In this case, the $R^2$ value for the linear relationship is only 0.41, indicating that the severity factor may not be an accurate indicator of hydrolysability since it does not factor in the acid concentration. Ramos and Saddler (1994) observed that for pre-treated Douglas-fir the $SO_2$ concentration was a significant indicator of the degree of hydrolysability. For the beetle-killed pine, however, the three substrates pre-treatments with lower $SO_2$ concentrations (2%, S4, S5, and S6), were hydrolysed as well as or better than substrates with higher $SO_2$. This may indicate that the effect of $SO_2$ may be variable and specific to the feedstock used.

Fermenting the water-soluble streams of all seven substrates resulted in four of the substrates fermenting similarly well (S1, S2, S3, S5), and the other three (S4, S6, S7) fermenting poorly (Figure 12). For those that actually did ferment, all of the sugar was consumed after 26 h, whereas only a third of the fermentable sugars were consumed in the same amount of time for the other three substrates. This could indicate that there was some kind of threshold concentration of inhibitory compounds above which fermentation will not proceed. It is likely that these inhibitory compounds are sugar degradation products such as furfural or HMF, which must be completely metabolized by yeast before any growth can occur (Taherzadeh et al. 2000).

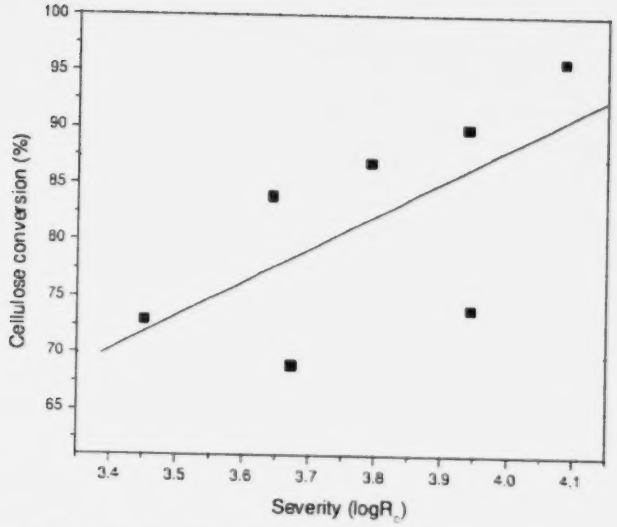



**Figure 11.** Relation of pre-treatment severity to the conversion of cellulose to glucose after 72 h of hydrolysis of the WIF produced after $SO_2$-catalyzed steam pre-treatment of beetle-killed pine at seven different conditions.

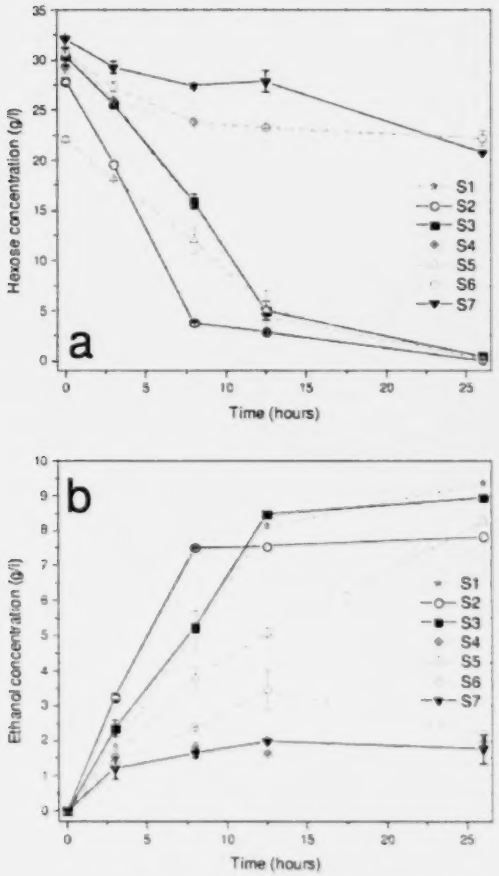



**Figure 12.** Hexose consumption (a) and ethanol production (b) during fermentation of the WSF of beetle-killed pine pre-treated at 7 conditions.

**Table 18**. Summary of sugar recovery, hydrolysis, and fermentation results obtained from beetle-killed pine pre-treated at 7 severities.

| Severity | Pretreatment severity ($logR_o$) | Hexose recovery (%) | Hydrolytic conversion[a] (%) | Fermentation conversion[b] (%) |
|---|---|---|---|---|
| S1 | 3.45 | 100 | 73 | 30 |
| **S2 (Low)** | **3.67** | **98** | **69** | **28** |
| **S3 (Medium)** | **3.64** | **96** | **84** | **37** |
| S4 | 3.79 | 93 | 87 | 7 |
| S5 | 3.94 | 90 | 74 | 29 |
| S6 | 3.94 | 90 | 90 | 10 |
| **S7 (High)** | **4.09** | **86** | **96** | **6** |

[a]**Percentage of pretreated cellulose converted to glucose after 72 hours of hydrolysis**
[b]**Percentage of hexoses converted to ethanol**

For substrates pre-treated at S1, S2, S3, and S5 conditions, the conversion of fermentable sugars to ethanol was between 29% and 37%, corresponding to 57%–73% theoretical ethanol yield (Table 18). These yields are slightly lower than expected, and might be due to inhibitory compounds.

Stenberg et al. (1998) found that in fermenting $SO_2$-catalysed, steam pre-treated mixed spruce and pine, pre-treatment temperature and not the overall severity factor affected substrate fermentability. This seemed to be the same case for our samples, with S1, S2, S3, and S5 samples pre-treated at 200°C or cooler, and S4, S6, and S7 pre-treated at 205°C or hotter. However, Stenberg et al. (2000) found that fermenting the water-soluble stream from substrates pre-treated at high severities (5.0) and temperature (230°C) still provided 78% of theoretical ethanol yield after 20 h, albeit with the addition of nutrients and supplemental glucose.

## 3.5 Hydrolysis of steam pre-treated substrates at decreased enzyme loadings

Steam pre-treated substrates were hydrolyzed at low enzyme loadings to determine if these substrates could be hydrolyzed effectively when the cellulase loading was decreased to the 5 FPU/g glucan level. As shown below, the hydrolysis of the substrates was compromised under conditions where the enzyme loading was decreased for the steam pre-treated beetle-killed substrates. Healthy lodgepole pine treated at the conditions described in the previous sections was also hydrolyzed using the 5 FPU/g glucan. Further pre-treatment optimization may be necessary to facilitate hydrolysis of steam pre-treated lodgepole pine substrates at enzyme loadings applied in this study (Figure 13).

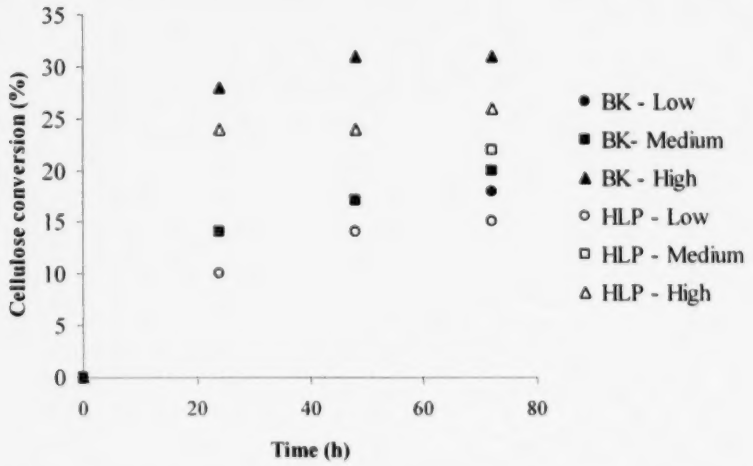



**Figure 13.** The enzymatic hydrolysis yields of steam pre-treated beetle-killed pine and healthy lodgepole pine substrates at cellulase loadings of 5 FPU/g glucan.

**Note:** All reactions were performed at constant reaction volume of 50 mL and a 2:1 ratio of β-glucosidase to cellulase activity (IU:FPU) at 2% consistency (%consistency= grams solid/grams+grams liquid), pH 4.8 in 50 mM sodium acetate buffer, 50°C and a shaker speed of 150 rpm. Substrates were pre-treated at conditions described previously.

## 3.6 Comparison of steam pre-treated beetle-killed lodgepole pine to steam pre-treated Douglas-fir

Softwood species vary significantly in their receptivity to steam pre-treatment (Schwald et al. 1989; Stenberg et al. 1998; Boussaid et al. 2000; Stenberg et al. 2000; Pan et al. 2005). For example, steam pre-treating spruce and *Pinus radiata* can result in reasonable sugar recovery and substrates that are readily hydrolyzed by cellulases (Stenberg et al. 1998, 2000). However, steam pre-treating Douglas-fir can lead to recalcitrant substrates forming which have required post treatments such as alkaline hydrogen peroxide to remove lignin after pre-treatment (Boussaid et al. 2001; Pan et al. 2004). Since the hydrolysis in the previous section indicated that applying low enzyme loadings caused minor differences among the substrates, and since previous work on Douglas-fir in Dr. Saddler's group has mostly been performed at higher enzyme loadings (20-60 FPU/g glucan range), the hydrolysis of Douglas-fir was compared to beetle-killed pine at the 20 FPU/g glucan loading with the wood pre-treated at the same conditions. The chemical compositions of steam pre-treated wood substrates prepared from Douglas-fir and lodgepole pine were similar (Table 19), with the beetle-killed pine substrate having slightly more glucose.

**Table 19**. Compositions of pre-treated Douglas-fir and beetle-killed pine pre-treated at identical steam pre-treatment conditions described in previous sections.

| Substrate | Conditions | Ara | Xyl | Glu | Gal | Man | AIL[a] | ASL |
|---|---|---|---|---|---|---|---|---|
| BKP | 200°C, 5 min, 4% $SO_2$ | 0.1 | 0.2 | 55 | 0.1 | 0.4 | 43 | 0.5 |
| Douglas-fir | 200°C, 5 min, 4% $SO_2$ | 0.0 | 0.0 | 49 | 0.1 | 0.4 | 45 | 0.5 |

Ara–Arabinan, Xyl–Xylan, Glu–Glucan, Gal–Galactan, Man–Mannan, AIL–acid incoluble lignin, ASL–acid soluble lignin. BKP–beetle-killed pine.

Upon hydrolyzing the two pre-treated softwood substrates from Douglas-fir and lodgepole pine in medium severity conditions, the latter was found to be more susceptible to enzymatic hydrolysis (Figure 14). The differences between the two substrates are difficult to surmise and will remain a focus of study of Dr. Saddler's research group within the coming year.

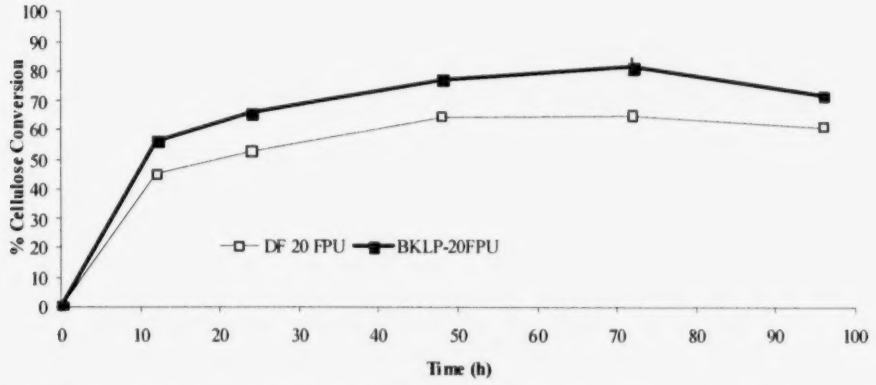



**Figure 14.** The hydrolysis of Douglas-fir and beetle-killed pine pre-treated at 200°C for 5 min and 4% $SO_2$ impregnation.

**Note:** All reactions were performed at a constant reaction volume of 50 **mL** and a 2:1 ratio of β-glucosidase to cellulase activity (IU:FPU) at 2% consistency (%consistency= grams solid/grams+grams liquid), pH 4.8 in 50 mM sodium acetate buffer, 50° C and a shaker speed of 150 rpm. Substrates were pre-treated at conditions described previously.

In recent years it has become increasingly apparent that understanding the features of a substrate that control its ease of hydrolysis by carbohydrate-degrading enzymes would greatly improve pre-treatment processes. Chemical properties aside, the pre-treatment–hydrolysis interface includes issues relating to macroscopic features such as particle size, to microscopic features such as the area actually accessible to cellulases, and to molecular-level factors such as cellulose crystallinity and degree of polymerization (Mansfield et al. 1999; Chandra et al. 2007). The ability to quickly elucidate factors imparted by pre-treatment that control hydrolysis may affect future pre-treatment developments. Performing cellulose hydrolysis on various substrates to test their ease of hydrolysis can be time-consuming and requires preparation and careful measurement of liberated carbohydrates. Therefore, we focused on developing and assessing techniques for measuring substrate parameters that could indirectly indicate the ease of hydrolysis. Techniques—such as water-retention value to measure fibre swelling, particle size analysis using the Fiber Quality Analyzer, and Simons' staining—have been used in the pulp and paper area with the objective of indirectly measuring the accessibility of the interior structure of fibres (Pan et al. 2007; Ogiwara and Arai 1969; Esteghlalian et al. 2001).Therefore, we adapted and applied these methods to characterize steam and organosolv pre-treated materials produced for subsequent hydrolysis by cellulases. Of note, we have focused extensively on the Simons' staining method which involves the differential adsorption of two dyes of varying size and affinity for cellulose. The orange dye is larger and has a higher affinity for cellulose than the blue dye. A substrate exposed to both dyes simultaneously will absorb the orange dye into larger substrate pores preferentially over the blue dye; however, if it has too few pores large enough to accommodate the orange dye, it absorbs the blue dye (Esteghlalian et al. 2001; Yu et al. 1995). In earlier work we found this method showed good potential for measuring the accessibility of cellulases to a kraft pulp (Esteghlalian et al. 2001), but it has yet to be applied to pre-treated substrates. For example, in the case of steam-pre-treated softwoods which are known to have many more small particles, it was thought likely that these substrates would be challenging to process. As a result, the work in this project focused on revisiting and streamlining the test to measure lignocellulosic substrates from beetle-killed pine pre-treated via the steam and organosolv processes. The initial results of modifying the Simons' staining test and relating the results of the test to the ease of hydrolysis of a set of pre-treated substrates has resulted in a publication and another subsequent publication in press on the application of fiber quality analysis, water retention measurements, and Simons' staining to steam and organosolv pre-treated lodgepole pine substrates.

# 4 Conclusions

The results indicate that lodgepole pine is a feedstock that shows great potential for use in biomass-to-ethanol schemes, though significant technical challenges remain. Further economic analyses may be required to determine the cost-effectiveness of technologies that may be part of a future bioconversion process. Both the steam and organosolv pre-treatment processes show potential for processing lodgepole pine wood chips. Organosolv pre-treatment results in cellulose-rich substrates that are readily hydrolyzed by cellulases. Steam pre-treatment also produces substrates which are amenable to hydrolysis. The high lignin content of steam pre-treated softwood substrates, such as those produced from lodgepole pine, may be an issue for hydrolysis at decreased enzyme loadings and at increased solids loadings, since the content of lignin in the reaction can range between 40%–50%. However, various wood species and samples such as Douglas-fir respond differently to steam pre-treatment when compared to lodgepole pine. The organosolv pre-treatment was able to process Douglas-fir wood at a level more consistent to that of beetle-killed pine wood compared to steam pre-treatment. Tests for assessing the potential ease of hydrolysis of cellulosic substrates have been relatively successful with the improvement of the Simons' staining method. This method enables further pre-treatment optimization after additional work confirming the ability of the test to predict the accessibility of pre-treated lignocellulosics to enzymatic hydrolysis.

Generally, the beetle-killed wood was more amenable to processing via organosolv pre-treatment and steam pre-treatment compared to the healthy lodgepole pine. This is most intriguing and may be related to other wood differences that affect processing, especially using steam pre-treatment. However, at this time, lodgepole pine shows promise for use as a biomass source for bioconversion processes.

# 5 Acknowledgements

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